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Professor Daniel H. Wreschner MD PhD

ANNUAL REPORT FOR AWARD NUMBER DAMD17-00-1-0451

Title: ANALYSIS OF THE SECRETED NOVEL BREAST-CANCER-ASSOCIATED MUC1/ZS CYTOKINE.

As described in our research proposal the *MUC1* gene is expressed in secretory epithelial tissues and at exceptionally high levels in human breast cancer cells. It is a well-known and widely accepted marker for breast cancer that can generate several functionally distinct MUC1 protein isoforms some containing a 20 amino acid tandem-repeat-array and additional forms lacking this domain. Findings emanating originally from our laboratory and subsequently confirmed by others, implicate the participation of the MUC1 proteins in signaling pathways. Analysis of MUC1 RT-PCR cDNA products revealed a supplementary and unique splice variant, MUC1/Zs, which is devoid of the tandem repeat array and utilizes a perfect out-of-frame splice acceptor site downstream to the tandem repeats. This splice event generates a secreted MUC1/Zs protein that harbors an N-terminal signal peptide and which contains a unique C-terminal stretch of 43 amino acids- this region shows marked homology with restricted regions of known cytokines and chemokines. We proposed to investigate the ***hypothesis that the MUC1/Zs protein is a novel, biologically important, cytokine*** **by (a)** analyzing MUC1/Zs protein expression in tumor tissues and correlating this expression with that of other MUC1 isoforms, **(b)** characterizing the signaling function of MUC1/Zs and proteins (receptor molecules?) with which the MUC1/Zs protein interacts, and **(c)** studying, both *in-vivo* and in *in-vitro*, the effect on tumor cell growth mediated by the MUC1/Zs protein.

(a) Analysis of MUC1/Zs expression in tumor tissues

In the first year of our work, we have focused on item (a) above. To analyze tumor expression we have generated, purified and characterized monoclonal antibodies that specifically recognize the MUC1/Zs isoform. The two most promising monoclonal antibodies, designated ZUM12D8 and ZUM7E7, were chosen for a more detailed analysis. Both these monoclonal antibodies were found to react specifically by western blotting with recombinant, bacterial MUC1/Zs- they did not react with any other MUC1 isoform thus indicating that they should be useful reagents for investigating MUC1/Zs expression in tumors and other tissues. The monoclonal antibodies, both of which are of the IgGγ1 subtype, were purified utilizing Protein A Sepharose chromatography following which they were concentrated using a Centricon apparatus with a molecular weight cut-off of 5kDa. Only the ZUM12D8 monoclonal antibody retained good quality activity following this procedure, and was thus used to generate both biotin-labeled and fluorescently labeled ZUM12D8 derivatives. Initial work carried out with the MUC1/Zs specific ZUM12D8 antibodies demonstrated that it could be used for immunohistochemical analyses, with the proviso that the sections used for staining were frozen sections and had been fixed with acetone and not with formaldehyde. As the *MUC1* gene is known to be expressed not only in breast tumor epithelial tissue but also in various other epithelial tissues (albeit at varying levels), we initially used the ZUM12D8 antibodies to stain frozen sections of skin as well as of tonsillar tissue (which contains pharyngeal epithelial cells).

This analysis demonstrated that the MUC1/Zs protein is indeed expressed in both these tissues, and in both cases, is restricted to epithelial regions. Within the pharyngeal epithelium of the tonsil and the epidermis of the skin, MUC1/Zs expression is restricted to the basal cells (basal keratinocytes in the skin). Within the crypt epithelium, MUC1/Zs is expressed by the majority of epithelial cells. This finding was interesting from two different aspects:

a) it indicated that MUC1/Zs expression is not restricted to breast epithelial cells, and
b) that it likely plays a function in skin epithelial cells.

We will now proceed to perform a more intensive immunohistochemical analysis with additional breast tumor and other tissues.

In our proposal we also wished to try to understand *the possible function of the MUC1/Zs molecule.* Clues to its function may be derived by looking at the functions of other proteins harboring partial homologies with MUC1/Zs. A tblastn search revealed that parts of the MUC1/Zs molecule showed the following homologies:

CD14_RAT MONOCYTE DIFFERENTIATION ANTIGEN CD14 PRECURSOR (LPS RECEPTOR,MYELOID CELL-SPECIFIC LEUCINE-RICH GLYCOPROTEIN).

```
0         80        160        240        320
|         |         |          |          |      |
_____________________________________________________
|  _____
```

Identities = 13/44 **(29%)**, Positives = 24/44 **(54%)**

```
MUC1/Zs:      33 APTTTKSCR----ETFLKCFCRFINKGVFWASPILSSGQDLWWY 72
                 +P T + C+    E  ++C+C F +    W+S +L +G+D+ +Y
CD14:         18 SPATPEPCELDQDEESVRCYCNFSDPQPNWSSAFLCAGEDVEFY 61
```

A DEFENSIN-LIKE PEPTIDE 1 (pdb|1B8W|1B8W)

```
  0         10         20         30         40
|         |          |          |          |  |
_____________________________________________________
|       ____________________________________
```

Identities = 10/28 **(35%)**, Positives = 13/28 **(46%)**

```
MUC1/Zs:      23 PSSTEK-NAIPAPTTTKSCRETFLK-CF 48
                 P   E  N +     T +CRE FL  C+
Defensin:      6 PRDCESINGVCRHKDTVNCREIFLADCY 33
```

Purothionin signature (PR00288) (Q39455)

```
Identities = 8/15 (53%)

MUC1/Zs:        8  KSCRETFLKCFCR
                     CR   LKCFCR
Purothionin:   59  GVCRGFPLKCFCRKP
```

Anaphylatoxin domain signature (PR00004) (CO4_MOUSE|P01029)

```
Identities = 7/12 (58%), Positives = 9/12 (75%)

MUC1/Zs:        38  SCRETFLKCFCR
                    +CRE FL C C+
Anaphylatoxin:      ACREPFLSC-CK
```

The common thread that weaves its way through these proteins, is that they all are secreted or membrane-associated proteins that participate in cell defense by functioning as antimicrobial proteins. Could it be that the MUC1/Zs secreted protein is also participating in such activities?

This possibility becomes all the more intriguing when one considers that mucins as a whole have long been considered to perform a protective function by acting as shields at the apical surface of secretory epithelial cells. It is tempting to speculate that the *MUC1* gene generates, on the one hand, a classical membrane-bound mucin protein that protects the cell, and on the other hand, generates by alternative splicing a secreted protein, MUC1/Zs, that protects the cell by harboring anti-microbial activity. We are presently investigating this possibility.

In addition to the above work, we were also interested in analyzing the presence of the MUC1/Zs protein in body fluids and especially in secretions from breast tumor tissues. To accomplish this we have established an ELISA assay that is specific for the MUC1/Zs protein. The ELISA assay developed is a sandwich assay in which a 96 well plate is coated with the MUC1/Zs specific monoclonal antibody ZUM7E7, followed by analyte application, and bound MUC1/Zs detected using biotinylated ZUM12D8, another monoclonal antibody which recognizes a different epitope on the MUC1/Zs protein. We have shown that this assay is specific for MUC1/Zs and shows a sensitivity down to 1ng/ml. We are now in the process of analyzing secretions from breast tumor tissues for the presence of the MUC1/Zs protein.